Research Note

A Probability Model for Enterotoxin Production of *Bacillus cereus* as a Function of pH and Temperature

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ABSTRACT

Bacillus cereus is frequently isolated from a variety of foods, including vegetables, dairy products, meats, and other raw and processed foods. The bacterium is capable of producing an enterotoxin and emetic toxin that can cause severe nausea, vomiting, and diarrhea. The objectives of this study were to assess and model the probability of enterotoxin production of *B. cereus* in a broth model as affected by the broth pH and storage temperature. A three-strain mixture of *B. cereus* was inoculated in tryptic soy broth adjusted to pH 5.0, 6.0, 7.2, 8.0, and 8.5, and the samples were stored at 15, 20, 25, 30, and 35°C for 24 h. A total of 25 combinations of pH and temperature, each with 10 samples, were tested. The presence of enterotoxin in broth was assayed using a commercial test kit. The probabilities of positive enterotoxin production in 25 treatments were fitted with a logistic regression to develop a probability model to describe the probability of toxin production as a function of pH and temperature. The resulting model showed that the probabilities of enterotoxin production of *B. cereus* in broth increased as the temperature increased and/or as the broth pH approached 7.0. The model described the experimental data satisfactorily and identified the boundary of pH and temperature for the production of enterotoxin. The model could provide information for assessing the food poisoning risk associated with enterotoxins of *B. cereus* and for the selection of product pH and storage temperature for foods to reduce the hazards associated with *B. cereus*.

Bacillus cereus is a gram-positive, spore-forming, widespread foodborne pathogen that is capable of producing an enterotoxin and emetic toxin that cause severe nausea, vomiting, and diarrhea *(8, 11)*. Two distinct foodborne disease types, diarrheal and emetic, are caused by *B. cereus*. The diarrheal-type illness mainly occurs in the Western hemisphere, whereas the emetic type is predominant in Japan *(4)*. Proteinaceous foods, sauces, desserts, vegetables, and dairy products are the most common vehicles of diarrheal illness, whereas pasta, fried rice, and cooked rice are most frequently linked to the emetic-type illness *(4, 12)*.

The first diarrheal-type disease caused by *B. cereus* was reported by Hauge *(6)* after the investigation of a hospital outbreak involving about 600 patients. The number of cases of foodborne illnesses attributed to *B. cereus* is increasing in industrialized countries. From 1993 to 1998, *B. cereus* was reported to cause 16.5% of the cases of foodborne illnesses in The Netherlands *(23)*. In 2003, *B. cereus* was isolated and confirmed in a case of home food poisoning in Belgium *(3)*. In 2007, *B. cereus* caused 19 outbreaks that resulted in 164 cases of illnesses, and the annual average number of reported foodborne outbreaks and the associated illnesses

were 10 and 130, respectively, in the United States *(2)*. Recently, the number of cases of foodborne illnesses caused by *B. cereus* has increased steadily in Korea. In Korea, foods such as kimbab (made from steamed white rice and rolled in dried laver seaweed) and tteok (a class of Korean rice cakes) are susceptible to contamination by *B. cereus* and are stored at room temperature before consumption. These types of food products are frequently implicated in foodborne illnesses caused by *B. cereus* poisoning. The Korea Food and Drug Administration reported 27 outbreaks of foodborne illnesses associated with *B. cereus* in Korea from 2001 to 2008. The majority of the cases were caused by enterotoxin; only one case was caused by emetic toxin *(9, 10)*. The number of cases of foodborne *B. cereus* illnesses is largely underestimated. The main reason is that the symptoms of the emetic illness are similar to those caused by *Staphylococcus aureus* poisoning, and the symptoms of the diarrheal illness are similar to those of *Clostridium perfringens* type A food poisoning *(20)*.

B. cereus can grow to large numbers in foods at room temperature *(9)*. Illnesses could occur after the consumption of foods that contain *B. cereus* cells at levels higher than 10^6 CFU/g, particularly when the foods are stored for a long period of time *(24)*. Beattie and Williams *(1)* reported that the formation of *B. cereus* enterotoxin was affected by the

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TABLE 1. *Treatments for examining the probability of toxin production of* Bacillus cereus *in TSB broth, the number of positive samples, observed and predicted probabilities of toxin production, and the final populations of* B. cereus

Treatment	Temp (°C)	pH	No. of positive samples	Observed P of toxin production (%)	Predicted P of toxin production (%)	B. cereus population (log CFU/ml)
1	15	5.0	0	0	0	3.0
2	15	6.0	0	0	0	3.6
3	15	7.2	0	0	2	4.1
4	15	8.0	0	0	2	3.9
5	15	8.5	0	0	1	3.7
6	20	5.0	0	0	1	4.8
7	20	6.0	4	40	33	5.5
8	20	7.2	7	70	77	6.4
9	20	8.0	7	70	69	6.3
10	20	8.5	6	60	47	5.7
11	25	5.0	8	80	83	6.9
12	25	6.0	10	100	99	7.3
13	25	7.2	10	100	100	7.8
14	25	8.0	10	100	98	7.6
15	25	8.5	8	80	94	7.1
16	30	5.0	10	100	100	7.5
17	30	6.0	10	100	100	7.5
18	30	7.2	10	100	100	7.7
19	30	8.0	10	100	100	7.5
20	30	8.5	10	100	99	7.5
21	35	5.0	10	100	100	7.6
22	35	6.0	10	100	100	7.6
23	35	7.2	10	100	100	7.7
24	35	8.0	10	100	100	7.7
25	35	8.5	10	100	99	7.6

pH of food products. Whereas *B. cereus* could grow at pH 4.5 to 9.0 *(14, 18, 21)*, the enterotoxin is produced at pH 5.0 to 9.0, with an optimal pH ranging from 7.0 to 8.0 for toxin production *(1)*. It has been reported that the enterotoxin was not stable at acidic conditions *(5, 19)*.

Numerous studies have been published relating to the growth or no-growth model of *B. cereus* and the detection of *B. cereus* toxin, but to our knowledge, there is no published literature reporting the effects of pH and temperature on *B. cereus* toxin production. The objectives of this study were to examine and model the probability of enterotoxin production of *B. cereus* in a broth model as affected by the broth pH and storage temperature.

MATERIALS AND METHODS

Preparation of *B. cereus* strains. Three strains of *B. cereus,* JNHE36 (Hbl, Nhe), ATCC 14579 (Hbl, Nhe), and F4810/72 (Nhe), were used in this study. Frozen stock cultures maintained at −70°C in tryptic soy broth (TSB; BD, Sparks, MD) containing 20% glycerol were thawed at room temperature, and 0.1 ml of each strain was inoculated separately into 10 ml of TSB and incubated at 35°C for 24 h in order to reach the stationary phase. Each cell suspension was centrifuged at 3,000 × *g* for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in 10 ml of 0.1% sterile peptone water (BD) by vortexing. This wash procedure was repeated twice. The three strains were mixed and diluted to approximately 5.0 log CFU/ml for use as an inoculum.

Sample preparation. A fractional factorial experimental design was used to select a subset of the experimental runs of a full factorial design to identify the probabilities of enterotoxin

production of *B. cereus* at broth pH of 5.0 to 8.5 and storage temperatures of 15 to 35°C. The ranges for pH and temperature were based on the reported boundary for production or no production of *B. cereus* enterotoxin *(1, 14, 18, 21)*. The pH of TSB was adjusted with 0.1 M HCl or 0.1 M NaOH to pH 5.0, 6.0, 7.2, 8.0, and 8.5. The pH-adjusted TSB (100 ml) was inoculated with 1 ml of *B. cereus* inoculum. The final concentration of *B. cereus* in the broth was approximately 3.0 log CFU/ml. Ten milliliters of inoculated TSB was put into sterile test tubes. The samples were stored at 15, 20, 25, 30, and 35°C for 24 h. A total of 25 combinations of pH and temperature (treatment, Table 1) were tested. Two trials with five replicate samples for each treatment in each trial were performed.

Detection of *B. cereus* enterotoxin. The presence of Nhe enterotoxin in each sample at the end of storage was assayed using a Tecra *Bacillus* diarrheal enterotoxin immunoassay kit (Bioenterprises Ltd., Roseville, New South Wales, Australia). The assay was performed according to the manufacturer's instructions. The toxin detection limit is 1 ng/ml. A sample with positive presence of *B. cereus* enterotoxin was recorded as a ''toxin production'' sample, otherwise as a ''no toxin production'' sample.

Model fitting. The probabilities of enterotoxin production of *B. cereus* (numbers of positive samples divided by the total numbers tested) for the 25 treatments as a function of the broth pH and storage temperature were fitted to the following equation, using the logistic procedure of SAS 9.2 for Windows (SAS Institute Inc., Cary, NC):

$$logit(P) = a_0 + a_1 \times T + a_2 \times pH$$
$$+ a_3 \times T \times pH + a_4 \times T^2 + a_5 \times pH^2$$

TABLE 2. *Parameter estimates of the logistic regression*[a]

Parameter	df	Estimate	95% CL		SE	Wald χ^2	$P > \chi^2$
Intercept	1	−114.0	−182.7	−45.2682	35.0711	10.5672	0.0012
Temp	1	3.3625	1.2800	5.4451	1.0625	10.147	0.0016
pH	1	19.7085	5.4619	33.9552	7.2688	7.3516	0.0067
Temp × pH	1	−0.1981	−0.4139	0.0176	0.1101	3.2404	0.0718
Temp × temp	1	−0.0248	−0.0581	0.00841	0.0170	2.1444	0.1431
pH × pH	1	−1.0701	−1.8302	−0.3099	0.3878	7.6128	0.0058

[a] Association of predicted probabilities and observed responses: concordant, 97.6%; discordant, 1.5%; and tied, 0.9%; *c*-value, 0.98. CL, confidence limits.

where P is the probability of toxin production, a_0 to a_5 are parameter coefficients, T is the incubation temperature (°C), and pH is the broth pH. The estimated probabilities of production of *B. cereus* enterotoxin (P) were calculated using the following formula:

$$\text{logit}(P) = \ln\left(\frac{P}{1-P}\right)$$

and the probability of toxin production is $P = 1/\left(1 + e^{-\text{logit}(P)}\right)$.

Assessment of goodness-of-fit. Model goodness-of-fit indexes, including the concordant, discordant, or tied rates, and the concordance index (c), which is equal to the area under the receiver operating characteristic curve, were used to determine the agreement between the predicted and observed values. The predicted values match the observed values when the concordant rate is 100%, the discordant and tied rates are 0%, and c is 1. The c value ranges from 0.5 (discriminating power not better than chance) to 1.0 (perfect discriminating power). The closer the c value is to 1, the better is the model prediction *(26)*.

RESULTS AND DISCUSSION

After incubation for 24 h, the final cell populations of *B. cereus* in all treatments ranged from 3.0 to 7.7 log CFU/ml. The numbers of toxin-positive samples and the final *B. cereus* counts for each treatment are listed in Table 1. Among the 25 treatments, six treatments had no toxin production, six treatments had some samples with toxin production, and 13 treatments had toxin production in 10 samples (Table 1). By comparing the final cell populations of *B. cereus* and toxin
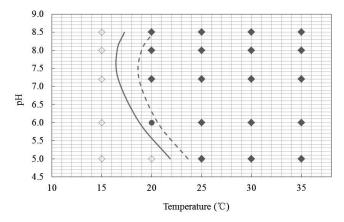

FIGURE 1. *Observed production (◆), likely production (●), or no production (◇) of* B. cereus *enterotoxin versus predicted interfaces for no toxin production (P ≤ 0.1, solid line) and toxin production (P ≥ 0.5, dashed line).*

production, it was observed that the enterotoxin of *B. cereus* could be reliably detected when the cell populations were >7.0 log CFU/ml, and the enterotoxin would most likely be detected at cell populations of 5.5 to 7.0 log CFU/ml. It is reasonable to assume that enterotoxin production is a result of the growth of *B. cereus*. Similar results have also been reported. Notermans and Blatt *(15)* reported that foods containing >4.0 log CFU/g *B. cereus* might not be safe for consumption. Beattie and Williams *(1)* reported that the enterotoxin of *B. cereus* was readily detectable in brain heart infusion broth or commercial food products after a 24-h incubation at 21°C, at which point the population of *B. cereus* reached >7.0 log CFU/ml.

The probabilities of toxin production were analyzed as a function of pH and temperature. The parameter coefficients, their standard deviations, and the associated statistics for the regression model are shown in Table 2. The probability model is:

$$\text{logit}(P) = -114.0 + 3.3625 \times T + 19.7085 \times \text{pH}$$
$$- 0.1981 \times T \times \text{pH} - 0.0248 \times T^2$$
$$- 1.0701 \times \text{pH}^2$$

Temperature, pH, and the quadratic term of pH significantly ($P < 0.05$) affected enterotoxin production. The percent concordance index of the model was 97.6%, indicating a high degree of agreement between the model predictions and the observed values. The c value was 0.98, indicating that the performance of the model was satisfactory. Generally, in studies related to growth and no-growth models of foodborne bacteria, P values ≤0.1 indicate an ''unlikely to grow'' or ''no-growth'' region, P values >0.5 indicate a ''likely to grow'' or ''growth'' region, and P values between 0.1 and 0.5 indicate an ''uncertainty'' region *(7, 13, 16, 22)*. Similar to the criteria for determining the growth or no-growth boundary, the probabilities of toxin production at P values ≤0.1 indicate a ''no enterotoxin production'' region, P values >0.5 indicate a ''toxin production'' region, and P values between 0.1 and 0.5 indicate a ''likely toxin production'' region. Using the model, the logit(P) values can be obtained, and the probabilities of toxin production of *B. cereus* for each treatment can be estimated (Table 1). Comparing the observed and predicted values (Table 1), the prediction underestimates the probability for treatment 7 (20°C and pH 6.0) and overestimates the probability for treatment 8 (20°C and

TABLE 3. *Comparison of the probabilities of enterotoxin production of* B. cereus *that are reported in selected food products and those obtained from the predictive model*

Type of substrate	Temp (°C)	pH	Observed P	Predicted P (%)	Source
BHI/milk[a]	17	5.8	0	1	van Netten et al. (1990) *(25)*
Minced meat	17	6.2	0	2	van Netten et al. (1990) *(25)*
Lasagna	17	5.8	0	1	van Netten et al. (1990) *(25)*
Rice meal	17	6.3	0	3	van Netten et al. (1990) *(25)*
Infant milk–based formulae	25	7.2	1	100	Rowan and Anderson (1997) *(17)*

[a] BHI, brain heart infusion broth.

pH 7.2). The observed probabilities for the other 23 treatments were in agreement with the predicted values, indicating that the model closely describes the probabilities of *B. cereus* enterotoxin production obtained from the experiment. In the present study, only a fixed storage time (24 h) was considered in the development of the probability model. It is possible that *B. cereus* can produce enterotoxin in a shorter or longer time than the 24-h time period. Additional studies are warranted to examine the incubation time as an influencing factor.

The comparison of the predictions for *B. cereus* toxin production and the data used for model development is shown in Figure 1. The predictions are close to the observations. The comparison indicates that the temperature-pH interface for *B. cereus* enterotoxin production is at 16 to 20°C and pH 5.0 to 8.5. At temperatures <25°C, the closer the broth pH is to pH 7.0, the higher is the probability of enterotoxin production. In addition, the production of enterotoxin of *B. cereus* occurs most readily at temperatures >25°C, regardless of the pH value. A similar result indicated that the largest amount of enterotoxin of *B. cereus* was produced at pH values 7.0 and 8.0 after 24-h incubation at 21°C in brain heart infusion broth *(1)*. The observed probabilities of toxin production of *B. cereus* in selected food products *(17, 25)* are listed in Table 3. The predictions from the model obtained from this study were in agreement with the reported probabilities, indicating the applicability of the model to these food products. Since there are limited independent data that can be used to fully validate the model, the use of the model needs further verification with the food products of interest.

In conclusion, the probability model presented herein describes the experimental data satisfactorily and identifies the temperature and pH limits for enterotoxin production of *B. cereus*. For food products that are likely to be contaminated by *B. cereus,* the model may be used to select pH and storage temperature that can prevent the production of enterotoxin by the pathogen.

REFERENCES

1. Beattie, S. H., and A. G. Williams. 2002. Growth and diarrhoeagenic enterotoxin formation by strains of *Bacillus cereus* in vitro in controlled fermentations and in situ in food products and a model food system. *Food Microbiol.* 19:329–340.
2. Centers for Disease Control and Prevention. 2010. Surveillance for foodborne disease outbreaks—United States, 2007. *Morb. Mortal. Wkly. Rep.* 59:973–979.
3. Dierick, K., E. V. Coillie, I. Swiecicka, G. Meyfroidt, H. Devlieger, A. Meulemans, G. Hoedemaekers, L. Fourie, M. Heyndrickx, and J. Mahillon. 2005. Fatal family outbreak of *Bacillus cereus*-associated food poisoning. *J. Clin. Microbiol.* 43:4277–4279.
4. Fratamico, P. M., A. K. Bhunia, and J. L. Smith. 2005. Foodborne pathogens: microbiology and molecular biology. Caister Academic Press, Norwich, England.
5. Granum, P. E., S. Brynestad, K. O'Sullivan, and H. Nissen. 1993. Enterotoxin from *Bacillus cereus*—production and biochemical characterisation. *Neth. Milk Dairy J.* 47:63–70.
6. Hauge, S. 1955. Food poisoning caused by aerobic spore-forming bacilli. *J. Appl. Bacteriol.* 18:591–595.
7. Hwang, C. A., and V. Juneja. 2011. Effects of salt, sodium pyrophosphate, and sodium lactate on the probability of growth of *Escherichia coli* O157:H7 in ground beef. *J. Food Prot.* 74:622–626.
8. Kim, J. B., J. M. Kim, S. H. Cho, H. S. Oh, N. J. Choi, and D. H. Oh. 2011. Toxin genes profiles and toxin production ability of *Bacillus cereus* isolated from clinical and food samples. *J. Food Sci.* 76:T25–T29.
9. Kim, J. B., J. M. Kim, C. H. Kim, K. S. Seo, Y. B. Park, N. J. Choi, and D. H. Oh. 2010. Emetic toxin producing *Bacillus cereus* Korean isolates contain genes encoding diarrheal-related enterotoxins. *Int. J. Food Microbiol.* 144:182–186.
10. Kim, J. H., E. G. Lim, H. C. Jang, J. Y. Park, S. J. Lee, M. S. Park, G. B. Choi, and B. K. Lee. 2009. A case of emetic toxin producing *Bacillus cereus* strains isolated from outbreak. *Korean J. Clin. Microbiol.* 12:48–52.
11. Kotiranta, A., K. Lounatmaa, and M. Haapasalo. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect.* 2:189–198.
12. Kramer, J. M., and R. J. Gilbert. 1989. *Bacillus cereus* and other *Bacillus* species, p. 22–70. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, New York.
13. Le Marc, Y., C. Pin, and J. Baranyi. 2005. Methods to determine the growth domain in a multidimensional environmental space. *Int. J. Food Microbiol.* 100:3–12.
14. Lindsay, D., V. S. Brozel, J. F. Mostert, and A. von Holy. 2000. Physiology of dairy-associated *Bacillus* spp. over a wide pH range. *Int. J. Food Microbiol.* 54:49–62.
15. Notermans, S., and C. A. Blatt. 1998. A risk assessment approach for food-borne *Bacillus cereus* and its toxins. *J. Appl. Microbiol.* 84:51S–61S.
16. Presser, K. A., T. Ross, and D. A. Ratkowsky. 1998. Modelling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration and water activity. *Appl. Environ. Microbiol.* 64:1773–1779.
17. Rowan, N. J., and J. G. Anderson. 1997. Maltodextrin stimulates growth of *Bacillus cereus* and synthesis of diarrheal enterotoxin in infant milk formulae. *Appl. Environ. Microbiol.* 63:1182–1184.
18. Rusul, G., and N. H. Yaacob. 1995. Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using Tecra-VIA and BCET-RPLA. *Int. J. Food Microbiol.* 25:131–139.
19. Spira, W. M., and J. M. Goepfert. 1975. Biological characteristics of an enterotoxin produced by *Bacillus cereus. Can. J. Microbiol.* 21:1236–1246.
20. Stenfors, A. L. P., A. Fagerlund, and P. E. Granum. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* 32:579–606.
21. Sutherland, A. D., and A. M. Limond. 1993. Influence of pH and sugars on growth and production of diarrhoeagenic toxin by *Bacillus cereus. J. Dairy Res.* 60:575–580.

22.   Tienungoon, S., D. A. Ratkowsky, T. A. McMeekin, and T. Ross. 2000. Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. *Appl. Environ. Microbiol.* 66:4979–4987.

23.   Tirado, C., and K. Schmidt. 2001. WHO surveillance programme for control of foodborne infections and intoxications in Europe, 7th report 1993–1998. FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and Zoonoses, Berlin.

24.   U.S. Food and Drug Administration. 1998. *Bacillus cereus* diarrheal enterotoxin, chap. 15. *In* Bacteriological analytical manual, 8th ed., rev. A. Available at: http://www.fda.gov/Food/ScienceResearch/Laboratory Methods/BacteriologicalAnalyticalManualBAM/ucm073688.htm. Accessed 26 June 2011.

25.   van Netten, P., A. van De Moosdijk, P. van Hoensel, D. A. Mossel, and I. Perales. 1990. Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *J. Appl. Bacteriol.* 69:73–79.

26.   Vermeulen, A., F. Devlieghere, K. Bernaerts, J. Van Impe, and J. Debevere. 2007. Growth/no growth models describing the influence of pH, lactic and acetic acid on lactic acid bacteria developed to determine the stability of acidified sauces. *Int. J. Food Microbiol.* 119:258–269.